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Prevention of Experimental Myointimal Hyperplasia by Immunomodulation

G. Stansby*[1], Y. C. Chan[1], C. S. Berwanger[1], S. Shurey[2], G. A. W. Rook[3] and J. L. Stanford[3]

[1]*Academic Surgical Unit, Imperial College School of Medicine at St. Mary's, London, U.K.,*
[2]*Department of Surgical Research, Northwick Park Hospital, Middlesex, U.K.,*
[3]*Department of Medical Microbiology, Windeyer Institute of Medical Sciences, Royal Free, and University College Medical School, London, U.K.*

Introduction: *we have tested the hypothesis that treatment with a mycobacterial preparation that modulates the antibody response, would diminish restenosis in a rat angioplasty model.*
Materials/Methods: *male Sprague–Dawley rats were used. All immunisations were given subcutaneously. Group A (control) received normal saline on days 0, 21, and 42. Group B received SRL172 on days 0, 21, and 42. Group C received SRL172 on days 0, 21, and 42, and hsp65/Incomplete Freund's on days 21 and 42. Group D received hsp65/ Freund's on days 21 and 42. Right common carotid arteries were balloon-injured on day 63 using a standard technique known to produce MIH and animals were sacrificed on day 77. For each carotid 6 μm cross sections were cut from paraffin blocks. Cross-sectional areas were measured by computerised planimetry.*
Results: *balloon injury resulted in MIH in all animals. Data represents mean ± SEM for the percentage of area enclosed within the internal elastic lamina occupied by MIH (% MIH); which for groups A, B, C, and D was 85 ± 11, 24 ± 3, 27 ± 7, and 17 ± 3 respectively. All the treatment groups had significantly less MIH when compared to the control group but no statistically significant difference was found between any of the treatment groups.*
Conclusions: *this is the first report that immunomodulation with mycobacterial material suitable for use in man, can reduce MIH. Since such modulation has low risk, this raises the prospect of an important new therapeutic modality to combat restenosis.*

Key Words: Myointimal hyperplasia; Restenosis; Immunotherapy; Myobacterium vaccae.

Introduction

Since its initial introduction, percutaneous transluminal angioplasty has become a successful and widely used treatment for patients with atherosclerotic disease in both the peripheral and coronary circulation.[1] However, restenosis after technically successful percutaneous transluminal angioplasty occurs in 25–30% of patients, limiting its long term efficacy.[2,3] Restenosis is usually due to myointimal hyperplasia (MIH) and the histologic appearance is independent of both the type and the location of vascular injury. The key initiating event appears to be vessel wall injury resulting in loss of endothelium and the production of cytokines and growth factors which produce a phenotypic change in the underlying vascular smooth muscle cells (VSMC).[4–8] A key factor may be diminished nitric oxide (NO) production by damaged endothelial cells, and several strategies that augment local NO release have beneficial effects on restenosis in animal models.[9]

The endothelial injury caused by angioplasty may be exacerbated by the host immune response to heat shock proteins (hsp). Hsps are proteins produced by stressed cells which have been implicated in the pathogenesis and the pathophysiology of various immunological disorders including atherosclerosis.[10] It is likely that they will be present on endothelial and smooth muscle cells in the region of an angioplasty. In effect the hsp acts as an autoantigen which can then be attacked by the immune system. In the vascular system this situation can be induced experimentally by immunising with a cross-reactive mycobacterial hsp (hsp65) which leads to endothelial damage in rabbits and mice.[10,11] The effect appears to be dependent on IL-4 secreted by Th2 (T-helper cell type 2) lymphocytes, and is probably mediated by antibody to the hsp.[11,12]

* Please address all correspondence to: G. Stansby, Northern Vascular Unit, Freeman Hospital, Newcastle upon Tyne, NE7 7DN, U.K.

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The relevance of these observations to man is suggested by the ability of affinity-purified human antibody eluted from hsp65 columns to damage stressed human endothelial cells *in vitro*.[12]

We have therefore tested the hypothesis that treatment with an appropriate mycobacterial preparation might alleviate restenosis via two related mechanisms; downregulation of IL-4 release, and modulation of the cross-reactive antibody response to hsp. The mycobacterial preparation selected (SRL172) potently downregulates pre-existing Th2 responses.[13] SRL172 is a heat-killed preparation of *Mycobacterium vaccae* (NCTC11659).

Materials/Methods

All experimentation was conducted in accordance with the Animals (Scientific Procedures) Act 1986, and all surgical procedures were performed in accordance with the guidelines of the U.K. Home Office. Carotid balloon injury was performed in adult male Sprague–Dawley rats (400–500 g). The rats were housed in an environmentally controlled facility on a light/dark (12/12 h) cycle and received standard laboratory chow (Special Diet Services, Essex, U.K.) and water *ad libitum*, except in the immediate (2 h) postoperative period.

The animals were divided into four groups (10 rats in each). All inoculations were administered subcutaneously (s.c.); saline and hsp65 emulsified in incomplete Freund's adjuvant were injected s.c. to the dorsal neck area, and SRL172 injections were given to the base of the tail. Group A (positive control) received 100 µl of normal saline on days 0, 21, and 42. Group B received 100 µl of a suspension of SRL172 on days 0, 21, and 42. Group C received 100 µl of SRL172 on days 0, 21, and 42, and 100 µl (50 µg) of hsp65 in incomplete Freund's adjuvant on days 21 and 42. Group D received 100 µl (50 µg) of hsp65 in incomplete Freund's adjuvant on days 21 and 42. In all animals, carotid balloon injury was then performed on day 63, and the animal sacrificed on day 77.

Material for injections

hsp65/Freund's

One hundred µg of hsp65 (*M. bovis* HSP65 kDa: batch MA18, GBF, Germany) dissolved in 100 µl of PBS (PBS Dulbecco's, GibcoBrl, Paisley, Scotland) was emulsified with 100 µl of incomplete Freund's adjuvant (Sigma, Steinheim, Germany).

SRL172 (Mycobacterium vaccae)

SRL 172 (heat-killed suspension of *Mycobacterium vaccae*) was obtained at 10 mg/ml (SR Pharma, U.K.) in vials of 0.3 ml. Each vial was diluted with 2.7 ml of sterile normal saline to produce a suspension of 1 mg/ml. Vaccination consisted of 100 µl containing 0.1 mg per rat.

Carotid balloon injury

In anaesthetised male Sprague–Dawley rats (400–500 g), the right common carotid artery was denuded of endothelium by the intra-luminal passage of a 2-Fr arterial embolectomy catheter (BVM Medical Limited, Leicester, U.K.), introduced through the left external carotid artery.[5] Briefly, rats were anaesthetised with intramuscular injections of xylazine hydrochloride (Bayer, Suffolk, U.K.) and ketamine hydrochloride (Pharmacia & Upjohn Limited, Crawley, U.K.). The ventral neck area was then shaved and prepped with aqueous povidone-iodine solution (Seton Healthcare Group plc, Oldham, U.K.). Under aseptic conditions, using a dissecting microscope with 5 × magnification, and with the use of micro-surgical set, through a midline neck incision extending from the mandible to the sternum, and after separation of the strap muscles in line with the skin incision, the ipsilateral sternocleiodomastoid muscle was removed with cautery. The external and common carotid arteries were then exposed, and a fine teflon sling passed under the external carotid artery. 7/0 ties (Ethicon, Edinburgh, U.K.) were applied to the distal external carotid artery, and a clip applied to the proximal external carotid artery. After arteriotomy with microscissors, the lumen of the external carotid artery was flushed with heparinised-saline. A 2-Fr arterial embolectomy catheter was then introduced into the lumen, and with controlled release of the proximal clip, it was passed proximally to the common carotid artery. The balloon catheter was then tied loosely in place with 7/0 sutures. The catheter was then passed three times with the balloon distended with 0.3 ml of air to generate slight resistance, and with the common carotid artery visibly distended. The balloon catheter was then removed, and the proximal external carotid artery ligated. Hemostasis was secured with cautery and wound closed in layers with 3/0 vicryl sutures (Ethicon, Edinburgh, U.K.) to subcutaneous tissues and to skin. The animals were then placed in a warm room and observed until full recovery.

At 14 days post-balloon injury (on day 77), the animals were sacrificed by an overdose of anaesthetic

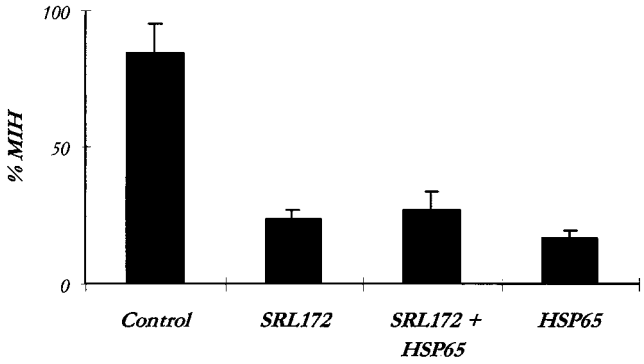

Fig. 1. MIH expressed as percentage area of MIH to the total area enclosed by the internal elastic lamina.

agent. The neck wound was reopened, and both carotid arteries were dissected free (together with harvest of thoracic aorta, through a ventral stenotomy). The entire length of the common carotid artery extending from the aortic arch to the carotid bifurcation was removed. The vessels were flushed with heparinised saline and then placed in 10% formalin-saline. All tissues were processed for paraffin wax embedding. For each sample, six 5 micron cross sections were cut from paraffin blocks and stained with haematoxylin and eosin (H&E), and mounted on glass slides.

Planimetric measurements were made directly from the slide with two independent blinded observers per section, using an interactive computerised image-analysis system (Seescan Biological Applications).

The ratio of the area of MIH to the total area enclosed by the internal elastic lamina (IEL) was calculated. This was then expressed as a percentage to represent the portion of the lumen occupied by the myointimal hyperplastic change[14] (Fig. 1).

Results were expressed as the mean $\pm$ SEM for the percentage of MIH (% MIH). Analysis of variance was performed for multiple comparisons. Mann–Whitney *U*-test was employed for inter-group comparisons, and the Bonferoni correction factor was used for multiple comparisons where appropriate. A p value of less than 0.05 was considered as a significant difference between the experimental groups.

Results

All animals survived the procedure but two died subsequently and were unavailable for analysis. Sections from six animals were deemed unsuitable for analysis due to poor orientation or thrombosis leaving eight carotids in group A, eight in group B, nine in group C and seven in group D available for analysis.

Injury to the common carotid artery had resulted in MIH in all animals. In contrast no MIH was detected in the un-angioplastied contralateral common carotid artery (data not shown). Results, expressed as the mean $\pm$ SEM for the percentage of MIH (% MIH) were 85 ± 11, 24 ± 3, 27 ± 7, and 17 ± 3 for groups A, B, C, and D respectively. Group A had significantly greater MIH when compared with Group B, C and D ($p = 0.007$, 0.003, 0.001 respectively), and no statistical significant differences exist between Groups B, C and D. (Fig. 1).

Discussion

MIH is a lesion consisting principally of VSMCs which have migrated from the media to the intima and proliferated there. To produce MIH VSMCs need to transform from a quiescent phenotype to one which is migratory, proliferative and secretory. Damage to the endothelium is thought to be a central event in initiating these changes and indeed MIH seems to be a universal response of blood vessels to endothelial damage. It complicates approximately 25–30% of angioplasties and vein bypass grafts and as such represents the major cause of angioplasty and vein graft failures.[4–8] It is a relatively rapid phenomenon, developing maximally by 14 days in the rat carotid and usually within 3 months clinically.[5] In this it is unlike atherosclerosis which usually takes many years to develop.

There is considerable evidence to suggest that the cytokines secreted by T-cells can modulate the pathological cellular events that occur in the development of atherosclerosis, where there is also VSMC migration and proliferation.[15,16] However, the role of the T-cell in MIH has been less well explored, although it has been demonstrated in an animal model that T lymphocyte depletion results in larger proliferative lesions after catheter denudation.[17]

Recent studies in our laboratory have shown that patients with atherosclerosis may have decreased Th1 immunity as compared to age-matched controls.[18] Whether this decrease is in any way causal was not clear. It did however lead us to postulate that the inflammatory responses to vessel injury might be potentially modifiable by altering the pattern of response. We have tested this hypothesis in the model reported here, and the reduction was substantial. If a similar effect could be produced in human restenosis it could have major therapeutic potential. Unfortunately, for technical reasons, we were unable to directly measure the T-cell cytokine response in rats. However, SLR172 is known to downregulate Th2 responses in other contexts.

There has been much interest in the role that Th1 and Th2 cytokines play in various other human diseases such as tuberculosis, asthma, rheumatoid arthritis and a number of autoimmune conditions.[19–21] Th1 cells produce IFN-γ, interleukin-2 (1L-2) and TNF. Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13.[22] Both types of T cell can cause cell-mediated inflammation, and both drive antibody formation, though different immunoglobulin subclasses are involved, and the quanitity of antibody accompanying a Th2 response is usually much greater. When regulation of Th1 responses fails, Th1-mediated autoimmune diseases such as multiple sclerosis can result.[23] Dysregulated Th2 responses can lead to a variety of pathologies, including allergic reactions, Th-2-mediated autoimmunity and also chronic fibrotic inflammation such as that seen in idiopathic pulmonary fibrosis (IPF).[24,25]

The existing literature suggests that endothelial damage mediated by recognition of hsp involves antibody and the antibody-stimulating Th2 cytokine IL-4.[11,12] Moreover patients with peripheral vascular disease have elevated levels of antibodies to hsp70.[26,27] It has been suggested that such antibodies may bind to stressed endothelial cells, and may be a factor in producing coronary artery disease after heart transplantation.[28] Mukherjee *et al.*[29] showed no association between preoperative antibody levels to hsp65 and coronary restenosis, but did show that those patients where levels of such antibodies dropped after angioplasty were less likely to restenose. In fact the role of antibodies to hsp could be complex, because patients with vascular disease have not only raised antibody, but also raised levels of the hsp themselves.[27] Thus an apparent fall in antibody levels may merely reflect an increase in levels of the protein. Moreover the hsp have regulatory effects, and bind to arterial smooth muscle cells, leading to enhanced survival wihout a requirement for internalisation.[30] Therefore the assumption that the effect of antibody is to damage cells which have membrane-associated hsp may only be part of the truth. Antibody may also block some essential regulatory role of the hsp.

In the light of these findings, it was anticipated that downregulation of Th2 responses by SRL172 (which contains mycobacterial hsp65) would limit MIH, whereas hsp65 in oil (group D), which enhances Th2 responses, was expected to increase the MIH. However the results indicate that the initial hypothesis was too simple, because both treatments were effective, and remained effective when used together (Group C), so a simple Th1/Th2 explanation is not possible.

An assessment of the literature on the effects of the Th1 cytokine IFN-γ on VSMC proliferation also reveals the inadequacy of the Th1/Th2 paradigm.[31] Frostegard *et al.*[32] reported that cytokine expression in advanced human atherosclerotic plaques was dominated by Th1 cytokines such as IFNγ, with Th1 cytokines in 70% of plaques using the sensitive method of PCR and in 30–50% using immunohistochemistry. Also in mouse models of atheroma formation and transplant vasculopathy the neutralisation or genetic absence of IFNγ markedly reduces the amount of VSMC related thickening that occurs.[33–37] Similarly, in the absence of leukocytes IFNγ can induce atherosclerotic changes in mice by an action on VSMCs to promote mitogenesis.[38] On the basis of these publications, downregulation of IFN-γ release by hsp65 in oil, with or without a switch to Th2, might be expected to be effective at limiting MIH.

In contrast, other studies have shown that IFNγ can inhibit VSMC proliferation *in vitro.*[31,39–43] Moreover, when infused into a rat carotid artery after balloon catheter denudation injury, IFNγ significantly reduced early smooth muscle cell replication and subsequent MIH.[44] Similarly inhibition of proliferation of VSMCs by 30–70% was seen when the gene for IFNγ was transfected into human endothelial cells co-cultured with VSMCs.[45] A protein has also been described which is upregulated by IFNγ in vascular smooth muscle cells and which is found to be downregulated in areas of anastomotic MIH.[46] In another study it was demonstrated that in histological studies of vein graft MIH in rats, IFNγ was found only in isograft controls. Downregulation of IFNγ appeared to accompany the early cytokine response triggering MIH.[47] Thus on the basis of this subset of publications, it might be expected that shifting the T-cell response towards Th1, with a resultant increase in IFNγ production, should prevent MIH.

Since the contrary conclusions can be drawn from the literature, it seems clear that the Th1/Th2 paradigm cannot by itself explain the pathogenesis of MIH. Indeed there are numerous other disease models where the greatest immunopathology is seen when a mixture of Th1 and Th2 cytokines is released into the lesion.[48–50] The results obtained in this study indicate that Th1/Th2 balance is also unable to explain the beneficial effects of the mycobacterial products used. There are two obvious unifying hypotheses, which are not mutually exclusive. First, it may be that SRL172 and hsp65 in oil are both able to alter the class or fine specificity of the antibody to hsp, so as to terminate a directly damaging effect, or relieve the blockage of a crucial regulatory role of hsp. The second hypothesis is that both treatments activate a class of regulatory cells that mediate a critical change in the pattern of cytokine

release. It has been pointed out elsewhere[51] that although hsp in oil has been used to terminate Th1-mediated autoimmunity such as adjuvant arthritis and streptococcal cell wall-induced arthritis by a mechanism thought to be the generation of a Th2 response,[52,53] the IL-4-secreting cells generated may be regulatory cells rather than Th2 effector cells.[51] Moreover such regulatory cells can also be induced by some initially Th1-inducing bacterial preparations (reviewed in [51]), and it is entirely possible that both treatments used in this study operate in this way.

It is important to note that in rabbits immunisation with hsp65 in adjuvant actually seems to provoke atheroma formation when combined with cholesterol feeding,[10] and similar hsp65 preparations can induce fatty streak formation in the aortas of mice.[11] In contrast, in the present study the material was protective against MIH. This may well reflect differences between atheroma and MIH. However hsp65 is in any case an unlikely candidate for therapeutic use in man, because quite apart from these effects on the vasculature, an increasing number of studies with the hsp itself, or with DNA vaccines encoding the hsp have led to autoimmune problems in animals.[54,55] On the other hand SRL172 appears to oppose autoimmunity in the situations where it has been tested,[56,57] and considerable long-term follow-up and safety data are available. Therefore clinical trials of this material in the prevention of restenosis could be considered if the same effect of reducing MIH could be shown in man.

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